IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re patent application of:

Rieping, *et al.*

Appl. No.: 10/817,431

Filed: April 5, 2004

Art Unit: 1652

Examiner: I. Chowdhury

Atty. Dkt.: 7909/84003

For: **Process for the Production of L-Amino Acids Using Strains of the Enterobacteriaceae Family**

Declaration Under 37 C.F.R. § 1.132

Commissioner of Patents
U.S. Patent and Trademark Office
Customer Service Window, **MS Amendment**
Randolph Building
401 Dulany Street
Alexandria, VA 22314

Sir:

The undersigned, **Dr. Rieping**, declares as follows:

1. I am, at present, **Research Scientist** at Degussa AG (hereinafter "Degussa") which is the assignee of all right title and interest in the above-captioned application. I am also named as an inventor on the application.

2. I have a Ph.D. in **Biology** from **University of Bielefeld** and have conducted research in the area of amino acid production by fermentation for **10 years**.

3. The research described herein was conducted under my supervision and I am therefore thoroughly familiar with the experiments that were performed and the results that were obtained.

4. The following study was conducted to examine the effect of overexpression of the yfiD open reading frame on bacterial L-lysine synthesis.

a) The L-lysine-producing E. coli strain pDA1/TOC21R is described in the patent application F-A-2511032 and deposited at the Collection Nationale de Culture de Microorganisme (CNCM = National Microorganism Culture Collection, Pasteur Institute, Paris, France) under number I-167. The strain and the plasmid-free host are also described by Dauce-Le Reverend *et al.* (*Eur. J. Appl. Microbiol. Biotechnol. 15*:227-231 (1982)) under the name TOCR21/pDA1.

b) After culture in antibiotic-free LB medium for approximately six generations, a derivative of strain pDA1/TOC21R which no longer contains the plasmid pDA1 is isolated. The strain formed is tetracycline-sensitive and is called TOC21R.

c) The strain TOC21R was transformed with the expression plasmid pTrc99AyfiD (see example 1 in the above-captioned application) and with the vector pTrc99A and plasmid-containing cells were selected on LB agar with 50 µg/ml ampicillin. Successful transformation can be confirmed after plasmid DNA isolation by test cleavages with the enzymes HindIII/XbaI and HpaI. The strains TOC21R/pTrc99 AyfiD and TOC21R/pTrc99A were produced in this way. Selected individual colonies were then multiplied further on minimal medium with the following composition: 3.5 g/l $Na_2HPO_4*2H_2O$, 1.5 g/l KH_2PO_4, 1 g/l NH_4Cl, 0.1 g/l $MgSO_4*7H_2O$, 2 g/l glucose, 20 g/l agar, 50 mg/l ampicillin.

d) The formation of L-lysine by the strains TOC21R/pTrc99AyfiD and TOC21R/pTrc99A was checked in batch cultures of 10 ml contained in 100 ml conical flasks. For this, 10 ml of preculture medium of the following composition: 2 g/l yeast extract, 10 g/l $(NH_4)_2SO_4$, 1 g/l KH_2PO_4, 0.5 g/l $MgSO_4*7H_2O$, 15 g/l $CaCO_3$, 20 g/l glucose and 50 mg/l ampicillin were inoculated and the batch was incubated for 16 hours at 37°C and 180 rpm on an ESR incubator from Kühner AG (Birsfelden, Switzerland). 250 µl of this

preculture was transinoculated into 10 ml of production medium (25 g/l $(NH_4)_2SO_4$, 2 g/l KH_2PO_4, 1 g/l $MgSO_4*7H_2O$, 0.03 g/l $FeSO_4*7H_2O$, 0.018 g/l $MnSO_4*1H_2O$, 30 g/l $CaCO_3$, 20 g/l glucose, 25 mg/l L-isoleucine, 50 mg/l ampicillin and 5 mg/l thiamine) and the batch is incubated for 72 hours at 37°C. After the incubation the optical density (OD) of the culture suspension is determined with an LP2W photometer from Dr. Lange (Berlin, Germany) at a measurement wavelength of 660 nm.

e) The concentration of L-lysine formed is then determined in the sterile-filtered culture supernatant with an amino acid analyzer from Eppendorf-BioTronik (Hamburg, Germany) by ion exchange chromatography and post-column reaction with ninhydrin detection.

f) The result of the experiment is shown in table 3.

Table 3

Strain	OD (660 nm)	L-Lysine g/l
TOC21R/pTrc99A	1.2	1.08
TOC21R/ pTrc99AyfiD	1.1	1.27

5. Based upon the results described above, it is my conclusion that L-lysine expression in Enterobacteria is increased in response to the overexpression of the yfiD open reading frame. This suggests to me that the effect of yfiD overexpression is not limited to any one L-amino acid.

6. I further declare that all statements made herein on the basis of personal knowledge are true, and all statements made on information and belief are believed to be true;

and further that any willful false statements or the like so made are punishable by fine or imprisonment or both under Section 1011 of Title XVIII of the United States Code; and that such willful false statements may jeopardize the validity of the above-captioned application or any patent issuing thereon.

Respectfully submitted,

09/13/2006
Date

Mechthild Rieping, Ph.D.